ONLINE SUPPLEMENT

EPOXYEICOSATRIENOIC ACID ANALOG MITIGATES KIDNEY INJURY IN A RAT MODEL OF RADIATION NEPHROPATHY

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Running title: Novel lipid-based mitigator of radiation kidney injury.

METHODS

Pressure-diameter studies with renal afferent arteriole

This study was carried out in a separate set of rats using *in vitro* perfused juxtamedullary nephron preparation described earlier [1,2]. Briefly, after pentobarbital anesthesia (50 mg/kg, i.p.) and midline laparotomy, the right renal artery was cannulated through the superior mesenteric artery, and the kidney was immediately perfused with a Tyrode's solution containing 6% albumin and a mixture of l-amino acids. After the microdissection procedures were completed the renal artery perfusion pressure was set to 100 mm Hg. The tissue surface was continuously superfused with a Tyrode's solution containing 1% albumin. After a 20-minute equilibration period, an afferent arteriole was chosen for study, monitored continuously by videomicroscopy, and baseline diameter was measured. The baseline afferent arteriolar diameter in Vehicle, 11Gy+Vehicle, 11Gy+EET-A and 11Gy+Captopril were 28.1±2.3, 27.2±1.1, 28.0±0.8 and 25.0±1.0μm, respectively. The relationship between diameter and perfusion pressure was determined. Perfusion pressure was varied in steps from 60 to 120 mmHg by adjusting the flow of gas in the perfusion reservoir. Measurements of afferent arteriolar diameter were made at 15 s intervals for a 5 min period at a single site at least 50 mm from any branch points. Steady state diameter was attained by the end of the second minute and the average diameter of the third through fifth minute at each perfusion pressure was used for statistical analysis. Finally, perfusion pressure was returned to 60 mmHg and a 5 min recovery period ensued. Renal afferent arteriolar pressure-diameter studies were carried out on samples collected at the end of the 12-week experimental protocol.

Urinary Biochemical Analysis

Urinary monocyte chemoattractant protein 1 (MCP-1) was measured using ELISA kit (BD Bioscience, San Diego, CA, USA). Urinary thiobarbituric acid-reactive substances (TBARS) concentrations were measured using the colorimetric OxiSelect TBARS assay kit (Cell Biolabs, San Diego, CA, USA), and urinary 8-isoprostane was measured by EIA (Cayman Chemical, Ann Arbor, MI, USA). Total nitric oxide production (from $NO_x = NO_3^- + NO_2^-$) was measured in urine using the Griess reaction [3] and by a commercially available assay kit (Cayman Chemical). Urinary levels of epoxygenase metabolites of arachidonic acid were determined using a LC-MS/MS method. In brief, Samples were stored at -80°C before analysis. Samples were warmed to room temperature, dried in a stream of nitrogen and the residue reconstituted in 40 μL of acetonitrile. Components were resolved on a 250 mm x 2.0 mm Kromasil C18-column packed with 5 μ diameter particles having 100Å pores. Gradient elution from 85% A to 15%A was used with eluant flow of 0.3 mL/min. Solvent A was water with 0.01% formic acid and solvent B was acetonitrile with 0.01% formic acid using the following profile: 15%B to 30%B in 10 min, 30%B to 60%B in 20 min, 60%B to 80%B in 15 min, hold at 80% for 5 min, then 20 min re-equilibration. MS/MS analysis was performed on an Agilent 6460 triple quadrupole mass spectrometer equipped with a Jet Stream™ interface. SRM was used to monitor oxidized arachidonate species in the negative ion mode. Precursor ion, product ion, collision energy and fragmenter voltage were optimized for each compound. Other parameters were as follows: drying gas flow=10 L/min at 325°C, nebulizer=20 psi, sheath gas flow=11 L/min at 325°C, capillary=3.5 kV, and nozzle=1.0 kV. Results acquired at unit-mass resolution.

Real-Time PCR analyses

Real-Time PCR analysis was carried out to assess the renal mRNA expressions of endoplasmic reticulum (ER) stress related signalling molecules glucose regulatory protein 78 (GRP78/BiP) and C/EBP-homologous protein (CHOP). Messenger RNA (mRNA) was isolated from kidney homogenate using RNeasy Mini Kit (QIAGEN, CA, USA) according to the manufacturer's instructions. The mRNA samples were quantified by spectrophotometry at 260 nm and 1 μg of total RNA was reverse-transcribed to cDNA using iScript™ Select cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The target gene expression was quantified by iScript One-Step RT-PCR Kit with SYBR green using MyiQ™ Single Color Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Each amplified sample in all wells was analyzed for homogeneity using

dissociation curve analysis using iQ5 Optical System Software, Version 2.1(Bio-Rad Laboratories, Hercules, CA, USA). After denaturation at 95°C for 2 min, 40 cycles were performed at 95°C for 10 s and at 60°C for 30s. Each sample was run in triplicate, and the comparative threshold cycle (C_t) method was used to quantify fold increase ($2^{-\Delta\Delta Ct}$) in the expression of the target genes compared to controls. In analyzing the relative expression of the target genes, the C_t values were normalized to two housekeeping genes (pgk1 and 18S). Statistical analyses were carried out for at least 5-7 experimental samples in each experimental group.

Isolation of renal micro vessels

Renal microvessels were isolated according to a method described previously [4]. Briefly, the kidneys were infused with a physiological salt solution composed of 0.1mM CaCl2, 125.0mM NaCl, 5.0mM KCl, 1.0mM MgCl2, 10.0mM glucose, 20.0mM HEPES (100μM Ca2+ PSS) and 6% bovine serum albumin and the renal microvessels were separated from the rest of the cortex with the aid of sequential sieving, a digestion period and collection under a stereomicroscope.

Western Immunoblotting

Fifty micrograms of homogenized kidney cortex protein samples were separated by SDS-PAGE on a 10% Tris-glycine gel, and proteins were transferred electrophoretically to a nitrocellulose membrane. Nonspecific binding sites were blocked by incubating the blots overnight at 4°C in a Tris NaCl buffer (TBS) containing 5% nonfat dry milk and 0.1% Tween 20. The primary antibodies used were for CYP2C23 (1:500), CYP2C11 (1:500), and soluble epoxide hydrolase (sEH) (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The blots were then washed in TBS-0.1% Tween and incubated with the horseradish peroxidase conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) for 1h. Detection was accomplished using enhanced chemiluminescence Western blot analysis, band intensity was measured densitometrically using ImageQuant TL 8.1 image analysis software (GE Healthcare, PA, USA) and the values were normalized to β-actin.

RESULTS

Renal expression of CYP2C11 and CYP epoxygenase metabolites were lower at 6-week post total body irradiation (TBI)

The expression of CYP2C11, the major EET producing enzyme in the kidney was lower at 6-week post TBI (11Gy). We demonstrated 45% lower expression of CYP2C11 in the kidney of TBI rats compared to control after 6 weeks of TBI. Accordingly, after 6 weeks of TBI, we also demonstrated a 30% lower urinary level of total epoxygenase metabolites in the TBI compared to control rats (Figure S1).

Hypertension and azotemia did not occur at 6-week post total body irradiation (TBI)

We demonstrated that rat received TBI (11Gy) did not develop hypertension and azotemia (elevated blood urea nitrogen) after 6 weeks of TBI. At 6-week post TBI, systolic blood pressure (TBI, 117±6 vs. Control, 121±8 mmHg) and blood urea nitrogen (TBI, 15.6±1.4 vs. Control, 17.2±4 mg/dL) were similar between TBI and control groups (Figure S2)

Afferent arteriolar response to elevations in perfusion pressure

We determined afferent arteriolar diameter response to increasing renal perfusion pressure from 60 mmHg to 120 mmHg. We demonstrate that in non-irradiated control rats an increase of renal perfusion pressure from 60 to 120 mmHg reduced renal afferent caliber and at 120 mmHg it was 87.91±1.12% of the baseline measured at 60 mmHg. But in irradiated rats the reduction of afferent caliber in response to renal perfusion pressure change was significantly less, and at 120 mmHg it was 98.38±0.95% of the baseline. However, in irradiated rats treated with EET-A or captopril, the reductions of afferent caliber in response to renal perfusion pressure change were greater than vehicle treated irradiated rats and similar to that observed in vehicle treated control rats (EET-A,

91.45±2.15% and captopril, 91.39±1.15%) (Figure S3).

Renal soluble epoxide hydrolase expression was not affected by total body irradiation

Renal mRNA and protein expressions of the EET metabolizing enzyme soluble epoxide hydrolase (sEH) were not affected by 11Gy total body irradiation (TBI) (Figure S4)

Renal oxidative stress did not occur at 6-week post total body irradiation (TBI)

The rats received TBI (11Gy) did not develop oxidative stress after 6 weeks of TBI. We demonstrated that at this time point, urinary TBARS, 8-isoprostane and nitrite/nitrate excretions were similar in TBI and control rats (Figure S5).

Radiation nephropathy was not associated with inflammation

Urinary monocyte chemoattractant protein 1 (MCP-1) excretion was not affected in irradiated rats (Figure S6).

ER stress is not involved in radiation nephropathy

In the present study, we investigated the contribution of ER stress associated apoptotic signaling in the kidney of irradiated rats after 12 weeks of TBI. We did not find any change in the renal mRNA expressions of ER stress associated apoptotic signaling molecules viz. GRP78 and CHOP in these rats (Figure S7).

FIGURE LEGENDS

Figure S1: (A) Renal protein expression of CYP2C11 was lower in the 11Gy+Vehicle group at 6-weeks post total body radiation (TBI). (B) At the same time point, urinary level of total epoxygenase metabolites of arachidonic acid was also lower in 11Gy+Vehicle compared to Vehicle group. In immunoblotting experiment, protein expression is normalized to the expression of β-actin. *P<0.05 vs. Vehicle, data expressed as mean ± SEM, and n=6 for each group.

Figure S2: (A) Systolic blood pressure and (B) blood urea nitrogen were similar between 11Gy+Vehicle and Vehicle groups at 6-weeks post total body radiation (TBI). P<0.05 vs. Vehicle, data expressed as mean ± SEM, and n=6 for each group.

Figure S3: Pressure-diameter relationships of renal afferent arteriole in different experimental groups. Experiments were carried out at the end of the 12-weeks protocol. The pressure-diameter relationship data are presented as the percent (%) change in the diameter from control diameter. P<0.05 vs. Vehicle, and #P<0.05 vs. 11Gy+Vehicle. Data expressed as mean ± SEM, and n=8 for each group.

Figure S4: Renal mRNA and protein expressions of soluble epoxide hydrolase (sEH) at 12-weeks post total-body radiation (TBI) compared to Vehicle. In immunoblotting experiments, protein expression of sEH is normalized to the expression of β-actin. *P<0.05 vs. Control, data expressed as mean ± SEM, and n=5 for each group.

Figure S5: (A) Urinary excretions of thiobarbituric acid-reactive substances (TBARS), (B) 8-isoprostane, and (C) nitric oxide (nitrite-nitrate) were similar between Vehicle and 11Gy+Vehicle groups at 6-weeks post total-body radiation (TBI). Data expressed as mean ± SEM, and n=6 for each group.

Figure S6: Urinary excretion of monocyte chemoattractant protein-1 was similar between the experimental groups at the end of 12-weeks protocol. Data expressed as mean ± SEM, and n=6 for each group.

Figure S7: Renal mRNA expression of endoplasmic reticulum stress markers (A) GRP78/BiP and (B) CHOP in different experimental groups. All measurements were done at the end of 12-weeks protocol. P<0.05 vs. Vehicle, and #P<0.05 vs. 11Gy+Vehicle. Data expressed as mean ± SEM, and n=8 for each group.

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